The Role of B Cells in *lpr/lpr*-induced Autoimmunity

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Summary

The primary roles of T cells and B cells in the initiation of systemic autoimmunity are unclear. To investigate the role of B cells, we crossed the "Jh knockout" mutation onto the autoimmune *lpr/lpr* background. Animals homozygous for both traits were obtained. As expected, these animals lack B cells. These animals also show no signs of autoimmune kidney destruction nor vasculitis, in spite of carrying the *lpr/lpr* mutation. In contrast, *lpr/lpr* littermates that had B cells had severe nephritis and vasculitis, as well as autoantibodies. These results demonstrate a primary role for B cells and/or (auto)antibodies in initiating several types of autoimmune-mediated tissue destruction. The implications of this finding for models and therapy of autoimmunity are discussed.

Systemic autoimmune diseases are the result of complex interactions among T cells, B cells, and target tissues. However, it has been difficult to distinguish the contributions of each cell type in both the initiation of the autoimmune response and the induction of specific pathologic lesions. This is in large part because the dependence of B cell activation on T cells and, reciprocally, the ability of B cells to act as potent APC for T cells.

Evidence of direct roles of T cells and B cells (or secreted Ig) in human lupus has been largely circumstantial. Examples include the presence of T cells in lesions or the presence of autoantibody at sites of inflammation (1–3). Animal models, on the other hand, have provided direct evidence for a role of both cell types (4–8). Intrinsic T cell defects have been clearly shown in *lpr* (9), BXSB, and NZB-based (10) models. Furthermore, in autoimmune MRL mice homozygous for the *lpr* mutation, thymectomy (11), anti-Thy1 (12), or anti-CD4 (13) antibody treatment ameliorates lymphoproliferation and delays autoimmune-mediated inflammation. T cells have also been observed within lesions of affected organs (14), most notably within the renal interstitium, surrounding vessels, and, to a lesser extent, glomeruli of MRL-*lpr/lpr* mice, coincident with the development of nephritis (8, 15).

There is also evidence of a primary role for autoantibodies and B cells in autoimmune pathogenesis. For example, some but not all anti-DNA antibodies cause nephritis upon injection into normal mice (6, 16, 17), whereas certain RF cryoglobulins (particularly of the IgG3 subclass) cause vas-

culitis and nephritis upon injection (18, 19). The role of B cells in initiating autoimmunity, perhaps by priming or expanding autoreactive T cells, is less clear. Using immunization of normal mice with cross-reactive variants of self-proteins, Lin et al. and Mamula et al. (20, 21) have suggested a key role for B cells in initiating T cell autoreactivity followed by a cascade of additional B cell autoreactivity. B cells from both *lpr/lpr* and NZB mice are intrinsically prone to autoantibody secretion (22–25). However, genetic studies have separated the secretion of certain autoantibodies from autoimmune manifestations, indicating that the role of B cells may be complex (26, 27).

Experiments using strategies to inactivate B cells have suggested their importance in autoimmunity. NZB.xid mice, which have developmentally arrested B cells owing to the xid defect, have a milder form of disease than their NZB counterparts (10). Similarly, C57Bl/6-*lpr/lpr* mice, treated with anti-IgM from birth, had few B cells and minimal glomerulonephritis; systemic vasculitis was unaffected (28). However, because anti-IgM treatment may have effects aside from reduction of B cells, genetic approaches would be helpful to determine whether Ig, B cells, or both are required for disease expression.

For this purpose, the *lpr/lpr* model of autoimmunity (29, 30) is ideal because fulminant autoimmunity and accumulation of abnormal T cells (31, 32) are controlled by a single recessive gene. Genetic studies are further facilitated by the recent discovery that *lpr* is a mutation in the *fas* gene (33).

The fact that the wild-type *fas* gene product transduces a signal for apoptosis (33, 34) has important implications for *lpr/lpr* autoimmunity. However, how a global *fas* defect causes autoimmunity remains unclear, in part because *fas* is expressed in many cell types and its regulation is activation dependent (35, 36). Thus, further work on the cellular contributions to autoimmunity in *lpr/lpr* mice is required.

To examine the role of B cells in *lpr*-induced autoimmunity, in the current studies we have used a novel genetic approach whereby *lpr/lpr* mice were deprived of B cells from birth without exogenous manipulation. We found that these *lpr/lpr* mice that lacked B cells failed to develop nephritis or vasculitis. Thus B cells and/or antibody play an important role in the initiation of a wide variety of autoimmune manifestations in *lpr/lpr* mice.

Materials and Methods

Mice

Jh knockout mice were produced as described (37) and had background genes of 129/Sv and C57Bl/6 origin. Mice carrying a single mutant Jh allele were intercrossed at an early stage of propagation, and homozygous mice were identified by PCR assay (see below). These mice were maintained usually by brother-sister matings. Henceforth, the mutated Jh allele will be referred to as JhD. F1 mice were produced from two separate matings of these homozygous JhD mice with MRL/*lpr/lpr* mice. The MRL-*lpr/lpr* mice were bred at Fox Chase Cancer Center (Philadelphia, PA) from an original breeding pair obtained from The Jackson Laboratory (Bar Harbor, ME). Three separate F2 crosses were set up at the same time. These mice were analyzed at 4.5–6 mo of age, as indicated in the tables and figures.

Genetic Testing

A dual-primer pair PCR assay was used to type for heavy-chain locus genotype. One pair of primers was specific for the *Neo* gene. These primers were Neo 5' (5'CCTTGCGCAGCTGTGCTCGAC-GTTG 3') and Neo 3' (5'GCCGCATTGCATCAGCCATGATGGA 3'). A second pair of primers amplified a region of the locus deleted by the gene targeting Jh 5' (5'GGACCAGGGGGCTCAGGTCAC-TCAGG 3') and Jh 3' (5' GAGGAGACGGTGACCGTGGTCCCT-GC 3'). Tail DNA preparation and PCR were carried out essentially as described (38). PCR buffer contained 2.5 mM MgCl$_2$. The following thermal cycler temperature program was used: 94°C 1-min initial denaturation; and then 30 cycles of 94°C 30 s/65°C 30 s/72°C 30 s, followed by 2 min at 72°C (MJ Research, Watertown, MA). Mice positive in both assays were considered heterozygotes, whereas mice positive in only one assay were considered homozygotes. FACS® analysis agreed with genotyping in all cases tested. A Southern blot assay was used to detect the *lpr* mutation, using the 180-bp Xba-RI fragment spanning the 5' end of the *fas* cDNA (33) as a probe. As expected, homozygous mutant mice had a smaller EcoRI fragment than wild-type mice, whereas heterozygotes had both bands at about half the homozygote intensity. Lymphoproliferation occurred in all mice typed as *lpr/lpr* with this assay with the exception of a single mouse (see Results). This mouse also lacked double negative T cells. Retyping by Southern blot clearly showed this mouse to be a heterozygote.

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Cell Preparation. Bone marrow (BM)[1] from a single femur was harvested from the first cohort of mice by flushing the marrow cavities with staining medium (RPMI 1640 deficient in biotin and lacking phenol red, supplemented with 2.5% FCS and 0.04% sodium azide). Spleens, thymi, and inguinal LN were harvested from all mice, and single-cell suspensions were prepared. After washing, ~5–20 × 10⁵ cells were included in staining reactions, which were performed as described (39). LN were weighed in both experiments. Spleens were weighed in the second experiment only; the large size of the spleens from the older *lpr/lpr* mice precluded disruption of entire spleens via our standard procedure. Therefore, a few representative portions were severed and disrupted.

Staining. The following mAbs were used in this study: 331.3 (40; anti-IgM), RA3-6B2 (41; anti-B220), 30H12 (42; anti-Thy1.2), and 53.7 (42; anti-Ly1.1). GK1.5 (43; anti-CD4) and 53.6 (42; anti-CD8) were prepared and kindly provided by N. Reutsch in the laboratory of M. Bosma (Fox Chase Cancer Center). 50AA12 (anti-CD3; PE conjugated) was purchased from PharMingen (San Diego, CA) and provided by N. Reutsch. Multicolor FACS® analysis was performed on a FACstar Plus (Becton Dickinson & Co., Mountain View, CA) equipped with dual lasers. 50,000 ungated events were collected. In all plots, dead cells were excluded from analysis by staining with propidium iodide. Percentages given of cells in various FACS® gates are of live cells.

Kidney Analysis

Histology. Kidney sections from each animal were analyzed by direct immunofluorescence and light microscopy, as previously described (6, 44). For direct immunofluorescence, fluoresceinated subclass-specific antisera were used, and the intensity of fluorescence was graded on a scale from 0 to 3+ for the presence and quantity of immune deposits (45). For light microscopy, one entire kidney from each animal was fixed in 10% formalin and embedded in paraffin. Multiple 4-μm sections through the center of the longitudinal axis of each whole kidney were obtained, and they were stained with hematoxylin and eosin.

Microscopic Evaluation. The sections were evaluated by one of us (M. P. Madaio) without knowledge of the donor mouse genotype. The severity of disease in each compartment (glomerular, interstitial, vascular) was graded on semiquantitative scoring of biopsy features (0–3+) according to previously described methods of Austin et al. (46), used in the analysis of human lupus nephritis. For purposes of grading in this study, 5–6-mo-old MRL-*lpr/lpr* mice with severe disease and 2-mo-old normal CBA/J mice were used for comparison (defined as 3+ and 0, respectively). Morphologic analysis involved assessment of the following: (*a*) for glomerulonephritis: glomerular hypercellularity (including glomerular cell proliferation, leukocyte exudation), karyorrhexis and fibrinoid necrosis, luminal occulusion, and cellular crescents; (*b*) for interstitial nephritis: infiltration of mononuclear cells, loss of normal architecture, and tubular necrosis; and (*c*) for vasculitis: perivascular infiltrates, intimal hyperplasia, and luminal occulusion.

ELISA for Ig and RF

ELISA assays were performed as previously described (38). Standard titrations were done on each plate, and concentrations were

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Table 1. *Analysis of Lymph Nodes*

Genotype*			4.5-mo-old mice					5.5–6-mo-old mice				
lpr	Jh	n	DN T cells‡	CD4+/CD8- T cells	Total cell count (×10⁷)§	LN weight	n	DN T cells	CD4+/CD8- T cells	Total cell count (×10⁷)	LN weight
						mg					*mg*
+/+	+/+	1	26	47	0.1	8	1	6	59	0.2	5
+/+	−/−	1	4	58	5.3	16	1	9	36	0.4	4
l/l	+/+	2	72 (12)	15 (5)	4.8 (4.1)	85 (48)	1	87	7	40	292
l/l	+/−	2	63 (19)	20 (8)	2.2 (1.5)	55 (30)	5	84 (4)	10 (2)	23 (9.6)	537 (277)
l/l	−/−	3	78 (12)	13 (7)	17 (9.1)	113 (41)	3	74 (6)	13 (2)	4 (1.6)	59 (34)

Numbers are percentages of live lymphocytes, rounded to the nearest whole number. When multiple mice have been analyzed in a group, the SEM is given in parentheses.
* Genotype was determined by Southern blot for *lpr* and PCR for Jh loci. +, wild-type allele; l, *lpr* mutant allele; −, inactivated Jh locus allele.
‡ DN T cell values are percentages of total T cells (as determined by CD3 or Thy1 expression in multicolor staining) to facilitate direct comparison of mice with and without B cells.
§ Total number of nucleated cells recovered from two inguinal LN.

estimated by comparison to curves fitted to standard values by use of a four-parameter curve fit on semilog plots.

Results

Analysis of F2 Progeny and Experimental Design. F2 mice provided all possible genotypic combinations. The genetic background of these mice was half MRL and half from the Jh background, which was itself a mixture of 129/Sv and C57Bl/6. Individual background genes segregate indepen-

dently of Jh and *lpr* types in such crosses and thus should not induce a bias between the groups, except those genes closely linked to the Jh and lpr loci themselves.

From 3 F1 males crossed to 9 F1 females, 81 F2 progeny were genotyped. The distribution of the nine possible genotypes (not shown) does not differ from the expected frequency ($p = 0.86$, χ^2, eight degrees of freedom). Two cohorts of mice, each containing three mice of the *lpr/lpr* JhD/JhD genotype along with randomly chosen individuals of other genotypes, were analyzed, the first at 4.5 mo and the second at

Table 2. *Analysis of Spleens*

Genotype*			4.5-mo-old mice					5.5–6-mo-old mice				
lpr	Jh	n	DN T cells‡	B220+/Thy1+	B220+/Thy1-	Total cell count (×10⁷)§	n	DN T cells	B220+/Thy1+	B220+/Thy1-	Spleen weight
											mg
+/+	+/+	1	12	1	59	7	1	29	21	44	77
+/+	−/−	1	13	1	3	6	1	11	7	3	53
l/l	+/+	2	56 (8)	22 (9)	43 (14)	33 (19)	1	68	37	25	605
l/l	+/−	2	40 (12)	16 (4)	43 (2)	27 (13)	5	68 (5)	28 (10)	20 (7)	1,475 (566)
l/l	−/−	3	64 (6)	39 (1)	7 (3)	6 (2)	3	67 (3)	42 (2)	4 (1)	108 (3)

Numbers are percentages of live lymphocytes, rounded to the nearest whole number. When multiple mice have been analyzed in a group, the SEM is given in parentheses.
* Genotype was determined by Southern blot for lpr and PCR for Jh loci. +, wild-type allele; l, lpr mutant allele; −, inactivated Jh locus allele.
‡ DN T cell values are percentages of total T cells (as determined by CD3 or Thy1 expression in multicolor staining) to facilitate direct comparison of mice with and without B cells.
§ Total number of nucleated cells recovered from the whole spleen. This was determined only for the 4.5-mo-old mice. Spleen weight was determined for the second cohort.

5.5–6 mo of age. In the first cohort, 12 mice were analyzed for autoantibodies and renal disease; nine were also subjects of FACS® analysis. In the second cohort, 14 mice were analyzed, 12 by FACS® as well. The data from the two cohorts are presented separately in the tables.

Peripheral Lymphoid Organs. Lymphadenopathy was observed in LN of all *lpr/lpr* mice (Table 1). Among the younger cohort of mice, lymphadenopathy (both weight and cell number) was similar regardless of whether mice lacked B cells. However, in the older cohort, lymphadenopathy was more severe among mice that had B cells. Spleens of the 4.5-mo-old *lpr/lpr* mice that had B cells contained ~5-fold more cells on average than mice heterozygous for *lpr* or +/+ mice (Table 2). Spleen weight averaged 15-fold higher in the older (5.5–6 mo) *lpr/lpr* mice with B cells. Thus, for splenomegaly, a difference was already apparent in the younger cohort, but it became more pronounced in the older cohort, whereas a difference in LN hyperplasia was apparent only among the older cohort of B cell–containing *lpr/lpr* mice. That lymphoaccumulation fails to progress with age in *lpr/lpr* mice lacking B cells suggests a role for B cells in augmenting the accumulation of *lpr/lpr* T cells.

Phenotypes of Peripheral Lymphoid Cells. *lpr/lpr* mice >4 mo are known to harbor large numbers of T cells with unusual phenotypes. The majority of these are Thy1$^+$/CD3^{dull}/B220^{dull}/CD4$^-$/CD8$^-$, termed double negative (DN) T cells (32). Lymphoaccumulation also includes an increase in the absolute numbers of CD4$^+$ and CD8$^+$ single positive (SP) cells, although to a lesser degree. Since B cells are required for efficient priming of T cells during immunization (47, 48), we investigated by FACS® whether the absence of B cells would affect T cell accumulation. As shown in Fig.
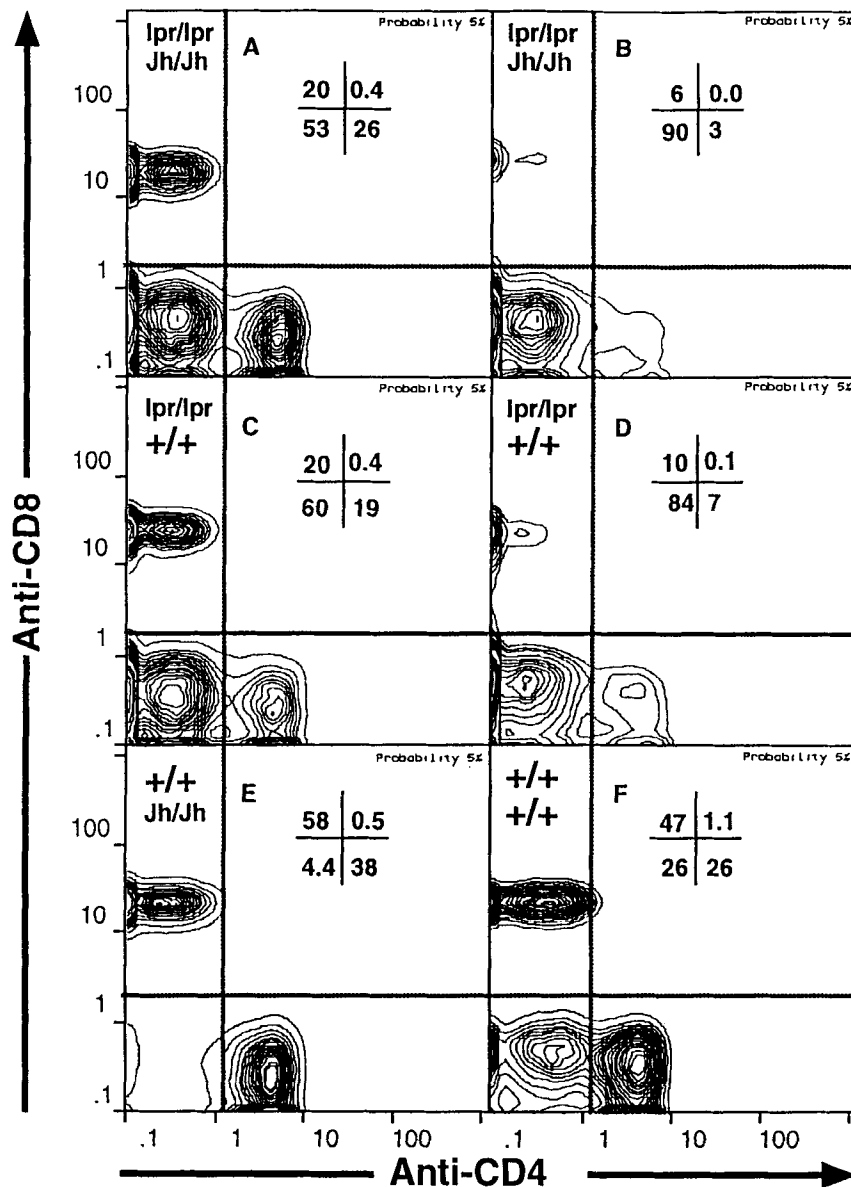

Figure 1. FACS analysis of CD4 and CD8 expression in CD3$^+$ LN cells of 4.5-mo-old mice. Single cell suspensions of inguinal LN were stained with fluorescein (FL)-anti-CD8, PE-anti-CD3, and allophycocyanin anti-CD4, followed by multicolor FACS® analysis. Shown are 5% probability plots from representative animals. Only live cells (unstained with propidium iodide) with forward and side scatter characteristics of lymphocytes that were positive for CD3 are shown. Genotypes of the animals are as follows: (*A* and *B*) *lpr/lpr* JhD/JhD; (*C* and *D*) *lpr/lpr* +/+; (*E*) +/+ JhD/JhD; and (*F*) +/+ +/+. Percentages of total CD3$^+$ cells in quadrants are given in each panel. Note the increased frequency of CD4$^-$/CD8$^-$ T cells in *lpr* mice. Animal-to-animal variability in DN T cell frequency is typical at this early stage of disease.

1 and Tables 1 and 2, absence of B cells had no effect on the proportions of these cells in LN or spleen. Whether defined as Thy1$^+$ or CD3$^+$ cells, there were no differences in the proportions of DN, CD4$^+$, or CD8$^+$ SP T cell subsets. In addition, there were similar frequencies of cells that were dull for Thy1, CD3, and B220 (Fig. 2 and Tables 1 and 2). Thus the presence of B cells does not affect the proportional nature of lymphoproliferation caused by the homozygous *lpr* mutation. However, because the number of accumulated cells was greater in *lpr/lpr* mice that had B cells, the absolute numbers of both DN and SP T cells were greater in such mice than in *lpr/lpr* mice lacking B cells. This was particularly evident in the oldest cohort of mice tested.

We also performed staining with anti-B220 and anti-IgM to enumerate B cells. As revealed by bright anti-B220 staining and (in the second cohort) anti-IgM staining, mature B cells were present only in those mice genotyped as heterozygous or wild type at the Jh locus (Fig. 2 and Tables 1 and 2). Some *lpr/lpr* mice with a wild-type Jh allele had a relatively small proportion of splenic B cells, presumably due to extensive T cell accumulation. However, in all such *lpr/lpr* mice, the absolute number of splenic B cells was increased.

Two curiosities were noted in the FACS® experiments. First, in some *lpr/lpr* JhD/JhD mice, a small population of B220^{dull} Thy1$^-$ cells was found in LN (data not shown). Three-color staining with anti-Thy1, anti-IgM, and anti-B220 staining in the second experiment showed that these cells were sIgM$^-$. Thus, these cells may have reflected seeding of the

LN by pre-B cells, which do develop in JhD mice (37, our unpublished data). However, similar cells have also been reported in previous studies of *lpr/lpr* mice; it was speculated that such cells are in the T lineage (49). This latter conclusion is consistent with the observation in separate staining reactions that nearly all lymphoid cells from the same LN were CD3$^+$ (data not shown). Further multicolor staining will be required to define the lineage of these cells.

Second, we noted in a single +/+ +/+ mouse a population of cells staining brightly for CD3 and B220 (Fig. 2 *D*). These B220$^+$/CD3$^+$ cells also stained with anti-IgM but not anti-Thy1. Thus, they probably represent B cells. The reason for the bright CD3 staining is unclear; it does not represent reagent interaction, as most B220$^+$ cells were negative for CD3. It is possible that a subpopulation of B cells expressed high levels of FcR, which bound the anti-CD3 antibody. Unfortunately, because of the experimental design, it was not possible to repeat the staining with different reagents and controls. Since cells falling in the B220^{bright} CD3$^+$ area were IgM$^+$ Thy1$^-$ in Fig. 2 *D* but IgM$^-$ Thy1$^+$ in Fig. 2, *A* and *B*, we gated on the B220^{dull} CD3^{dull} population characteristic of DN T cells to quantitate comparable cell populations in each mouse.

Central Lymphoid Organs. Single-cell suspensions derived from thymi and BM were examined by FACS®. Similar quantities of thymic cells were recovered from all mice. *lpr/lpr* mice with B cells had higher proportions of CD4$^-$/CD8$^-$ thymocytes than *lpr/lpr* mice that lacked B cells (Fig. 3 and
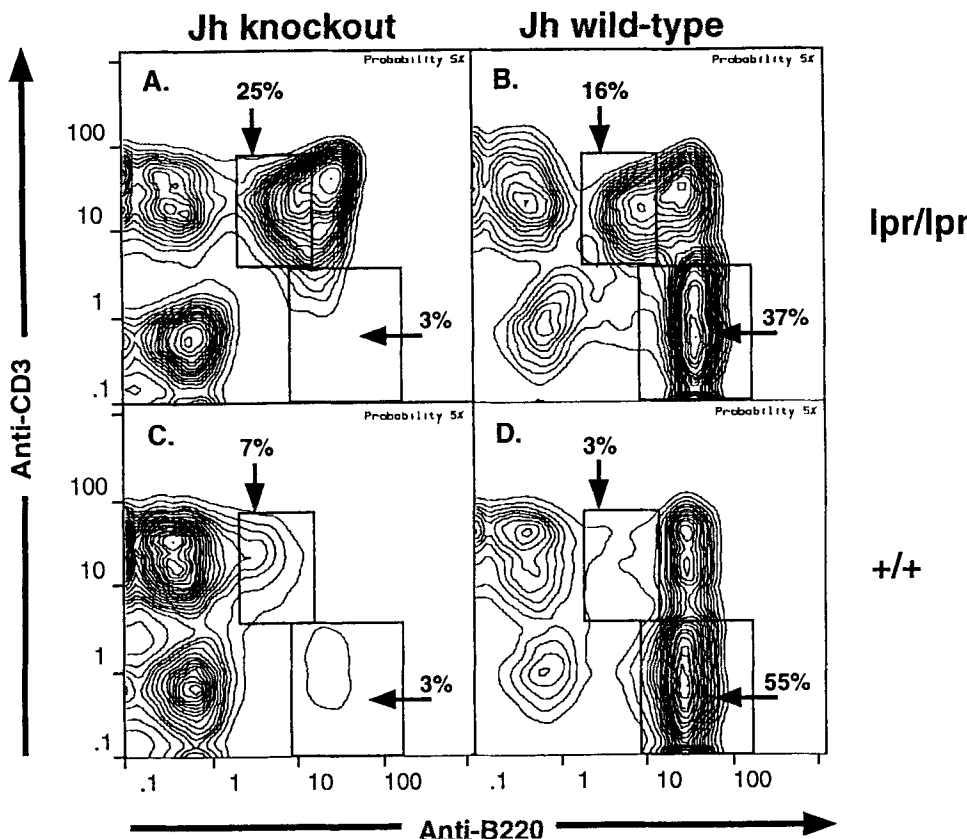

Figure 2. FACS® analysis of B220 and CD3 expression on splenocytes of 5.5-mo-old mice. Cells were stained with FL-anti-B220 and PE-anti-CD3. Gating and plots are as in Fig. 1. The *lpr* and Jh genotypes of the animals are as follows: (*A*) *lpr/lpr* JhD/JhD; (*B*) *lpr/lpr* JhD/+; (*C*) +/+ JhD/JhD; and (*D*) +/+ +/+. Note the virtual absence of B220^{bright}/CD3$^-$ B cells (lower right box in each panel) in the JhD/JhD animals (*A* and *C*). The upper left box in each panel shows a population of B220^{dull}/CD3^{dull} cells. These cells are rare in +/+ mice (*C* and *D*) but are common in *lpr/lpr* mice (*A* and *B*), and accumulate in similar proportions regardless of the Jh genotype (compare *A* and *B*). The population of cells brighter for both CD3 and B220 seen in *A* and *B* likely comprises an additional abnormal population of T cells. See text for details. The 3% of B220$^+$ cells in *A* and *C* do not express IgM (not shown) and thus resemble pre-B cells.

A

11	74
2.2	12

B

10	53
23	13

C

50	33
5	10

D

13	72
4	13

Anti-CD8 (vertical axis): 100, 10, 1, .1

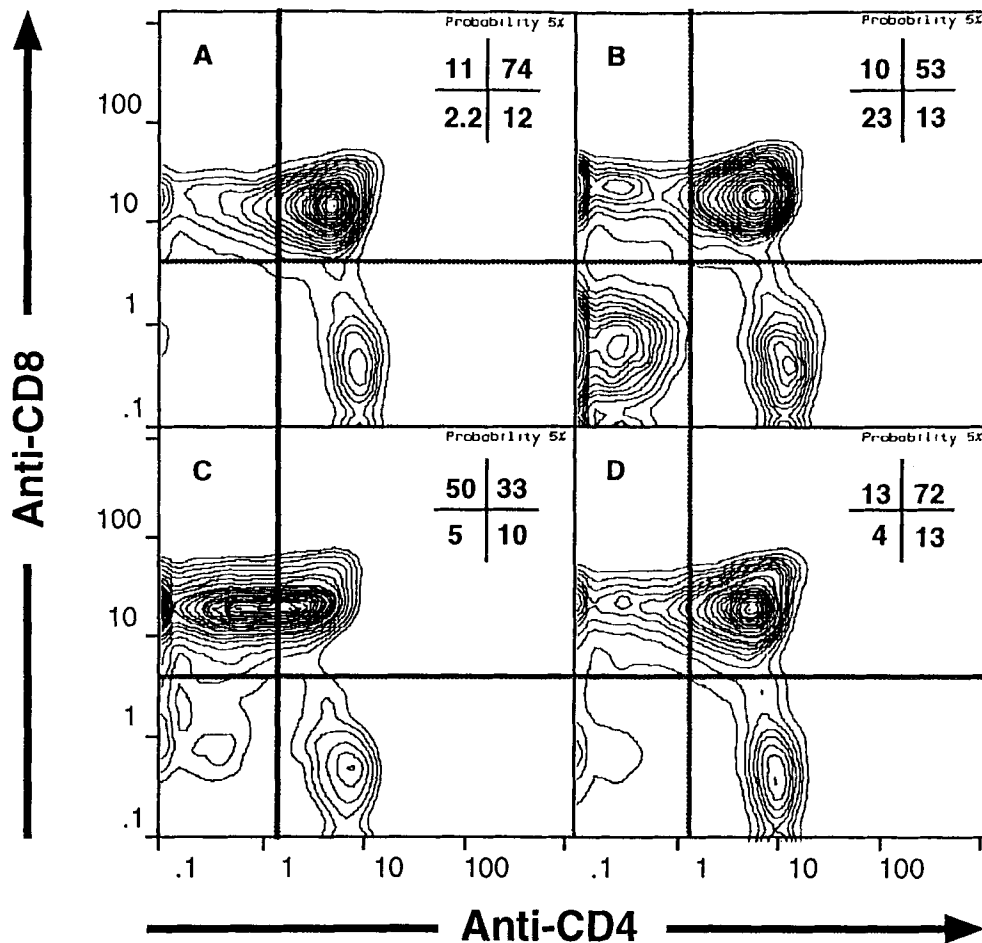**Anti-CD4** (horizontal axis): .1, 1, 10, 100

Figure 3. FACS analysis of CD4 and CD8 expression among Thy1$^+$ thymocytes of 4.5-mo-old mice. Single-cell suspensions were stained with biotin anti-Thy1, FL-anti-CD8, and allophycocyanin-anti-CD4, followed by Texas Red-avidin. Gating and plots are as in Fig. 1. Percentages of total Thy1$^+$ cells are given for each quadrant. In all thymi, >96% of live lymphocytes were Thy1$^+$. The *lpr* and Jh genotypes of the animals are as follows: (*A*) *lpr/lpr* JhD/JhD; (*B*) *lpr/lpr* +/+; (*C*) +/+ JhD/JhD; and (*D*) +/+ +/+. Note the large fraction of DN Thy1$^+$ cells in the *lpr/lpr* +/+ animal (*B*) not present in any of the other genotypes. The thymi of the mice used for *A* and *B* yielded similar numbers of total cells.

Table 3). The proportion of DN thymocytes in *lpr/lpr* mice with B cells was also higher than in +/+ littermates with or without B cells. The nature of these excess DN thymocytes, which have been previously observed in *lpr/lpr* mice, is somewhat uncertain (49–51). They may represent DN abnormal T cells, which were also found in larger numbers in peripheral lymphoid organs of the older *lpr/lpr* mice that had B cells. It is unlikely that these are B cells since thymic profiles

Table 3. *Analysis of Thymus*

Genotype*			4.5-mo-old mice				5.5–6-mo-old mice			
lpr	Jh	n	DN T cells	CD4$^+$/ CD8$^-$ T cells	CD8$^+$/ CD4$^-$ T cells	CD4$^+$/ CD8$^+$ T cells	n	DN T cells	CD4$^+$/ CD8$^-$ T cells	CD8$^+$/ CD4$^-$ T cells	CD4$^+$/ CD8$^+$ T cells
---	---	---	---	---	---	---	---	---	---	---	---
+/+	+/+	1	4	13	11	72	1	2	10	6	82
+/+	-/-	1	5	10	50	33	1	2	10	7	82
l/l	+/+	2	15 (8)	15 (2)	15 (5)	55 (1)	1	26	23	8	41
l/l	+/-	2	19 (12)	23 (13)	19 (7)	39 (32)	5	34 (9)	21 (3)	11 (1)	33 (9)
l/l	-/-	3	2 (0)	14 (2)	12 (0)	71 (2)	3	3 (0)	13 (1)	10 (2)	74 (2)

Numbers are percentages of live lymphocytes, rounded to the nearest whole number. When multiple mice have been analyzed in a group, the SEM is given in parentheses. Percentages are of T lymphocytes as determined by gating on Thy1 as a third color.
* Genotype was determined by Southern blot for lpr and PCR for Jh loci. +, wild-type allele; l, lpr mutant allele; -, inactivated Jh locus allele.

were gated for the expression of Thy1 (which included usually >95% of live cells). The significance of the B cell dependence of DN thymic T cells is discussed below. BM staining for B220 and anti-IgM was performed on the first cohort of mice and revealed no differences in the proportions of pre-B (i.e., B220^{dull}/IgM$^-$) or B (B220$^+$/IgM$^+$) phenotype cells between *lpr/lpr* and heterozygous or +/+ mice (data not shown). As expected, mice homozygous for the JhD mutation lacked mature (B220^{bright}) or immature (B220^{dull}) sIgM$^+$ B cells, although most had detectable pre-B cells (data not shown).

Autoantibody Production. Serum RF activity as well as serum IgG1 and κ levels were measured in all mice (Fig. 4). As expected, mice that lacked B cells lacked serum RF and Ig. *lpr/lpr* animals with B cells had markedly elevated Ig and RF levels compared with mice with B cells but homozygous for *lpr*. Elevations of RF (two to five orders of magnitude compared with non-*lpr* controls) were proportionally much greater than general hypergammaglobulinemia (about one order of magnitude of elevation; see Fig. 4), consistent with antigen-selective autoantibody production in *lpr/lpr* mice (52). Thus,

age-related *lpr/lpr*-controlled autoantibody production resembling that of MRL-*lpr/lpr* mice had ensued among the cohorts of F2 mice.

Nephritis and Vasculitis. Renal pathology was interpreted without knowledge of mouse genotypes. The mice could be readily distinguished into two groups with strikingly different morphologies: a group with normal architecture and no immune deposits and a group with severe glomerulonephritis, interstitial nephritis, vasculitis, and both glomerular and interstitial immune deposits. Representative examples are illustrated in Fig. 5. Details of the pathology are described in the legends. Severe nephritis always segregated with the presence of both the *lpr/lpr* genotype and B cells, and this was observed for all animals in both cohorts (a total of 10 *lpr/lpr* mice with B cells). By contrast, none of the six *lpr/lpr* mice that lacked B cells and circulating Ig developed nephritis; their histology was indistinguishable from the four +/+ littermates that had B cells and with normal control CBA/J mice.

Discussion

To further elucidate the role of B cells in MRL-*lpr/lpr* mice, a series of F2 mice were created from crosses of *lpr/lpr* homozygotes and mice homozygous for a mutation (JhD) that prevents development of mature B cells. Strikingly, in the absence of B cells, *lpr/lpr* mice developed neither glomerulonephritis, vasculitis, nor interstitial nephritis. In contrast, all of the *lpr/lpr* littermate mice with B cells developed severe nephritis and autoantibodies. These results indicate that B cells and/or autoantibodies play a primary role in the initiation of lupus nephritis in this genetic background. Severe nephritis was present in B cell–containing *lpr/lpr* mice at 4 mo of age, a time when lymphoaccumulation was equivalent in mice lacking B cells and animals with B cells. Thus, suppression of nephritis was not the result of decreased lymphoaccumulation. For each form of nephritis observed in B cell-sufficient mice, it is formally possible that the role of B cells is as (auto)antibody-forming cell precursors, as APC in causing T cell activation, or both. However, likely roles of B cells for each form of nephritis are suggested by previous work on pathogenesis.

The requirement of B cells for induction of glomerulonephritis substantiates previous work suggesting a role of immune deposits in glomerulonephritis (1, 5, 6, 16–18, 45) and indicates that immune deposit formation is likely a pivotal event in the initiation of the glomerular lesion. Furthermore, although other systemic and local cellular and cytokine perturbations are present in these animals (53, 54), they are by themselves insufficient to induce even microscopic disease. In this regard, it will be of particular interest to evaluate cytokine levels within the glomeruli of mice lacking B cells to determine the influence of B cells and Ig deposition on cytokine disregulation reported to occur before the onset of overt disease (54).

Absence of interstitial nephritis in *lpr/lpr* mice lacking B cells is also interesting and surprising, particularly because
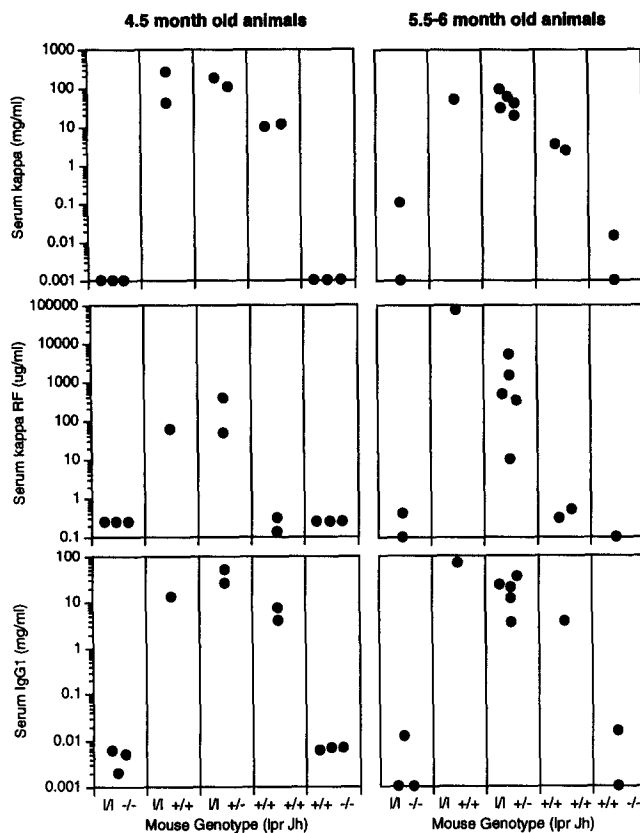

Figure 4. Serum Ig and RF levels. Levels of serum κ (*top*), κ-positive anti-IgG2a (*middle*) and IgG1 (*bottom*) were determined. The different age cohorts are represented separately (*left* and *right* columns). Each point represents a single mouse. Mice are divided by *lpr* or *Jh* genotype, which is given at the bottom of the figure. *l/l*, *lpr/lpr*; or +/+, wild type; –/–, homozygous Jh deletion; +/+, homozygous wild type; +/–, heterozygotes.
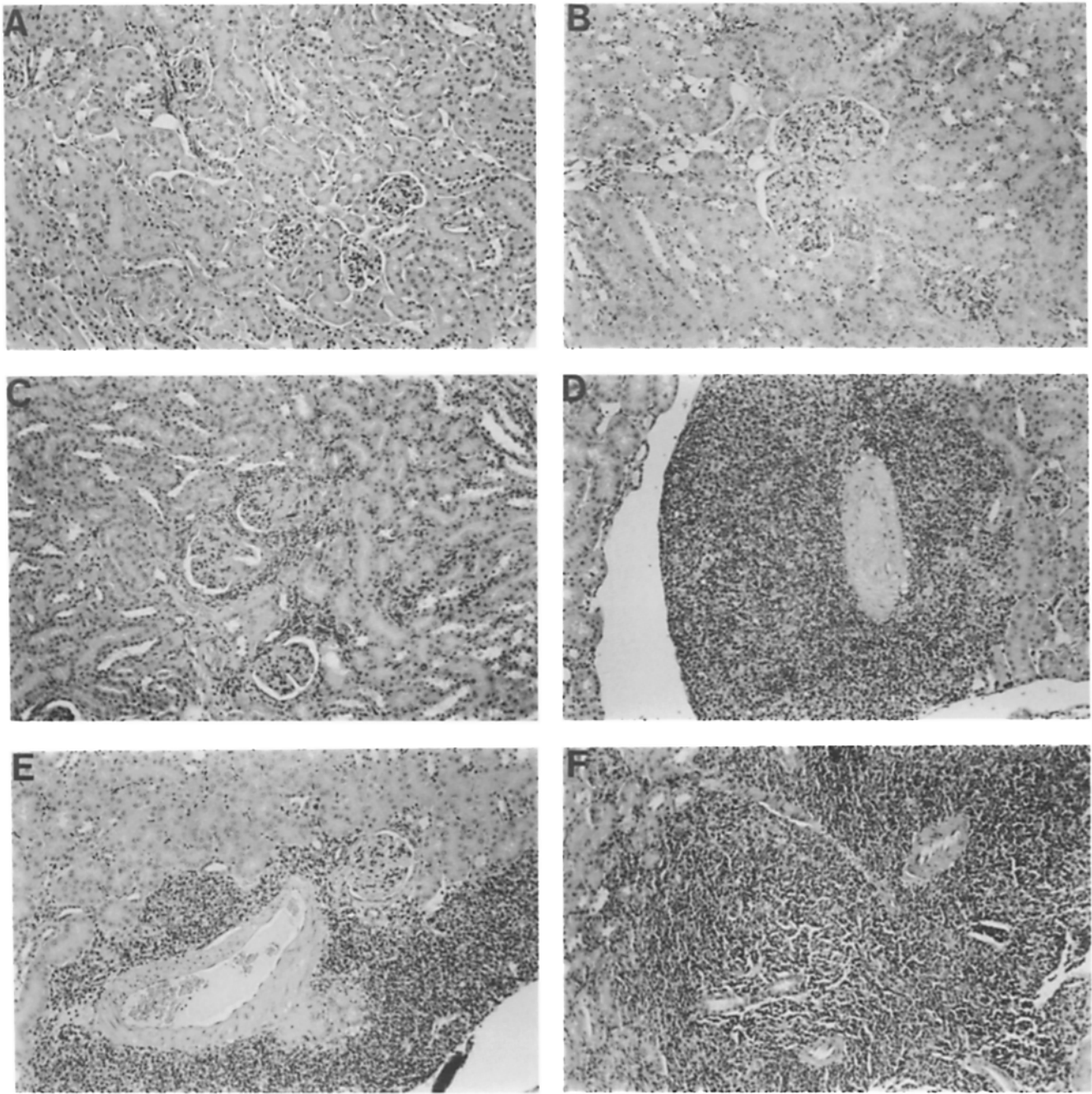
Figure 5. Histologic evaluation of kidneys from *lpr/lpr* mice (hematoxylin and eosin stained). Representative sections are presented to illustrate severe glomerulonephritis, vasculitis, and interstitial nephritis, which were present in all *lpr/lpr* mice with B cells in both age groups (*n* = 10). (*A*) *lpr/lpr* mouse without B cells at 5.5 mo of age (×400). The histology is normal. (*B–F*) *lpr/lpr* mice with B cells (×400). (*B* and *C*) Proliferative glomerulonephritis with mild interstitial hypercellularity. (*D*) Perivasculitis (intense). (*E*) Glomerulonephritis, perivasculitis, and interstitial nephritis. (*F*) Severe interstitial nephritis with tubular necrosis.

of the hypothesis that T cells and macrophages are the primary initiators (4, 55). It has been reported that intense cellular inflammation often precedes Ig deposits along tubular basement membranes (56). In addition, T cell lines have been isolated that are nephritogenic upon injection (15). Our results thus suggest that B cells may be required for the activation of autospecific T cells that infiltrate the kidney. Local cytokine

release due to glomerular inflammation could also be required to promote interstitial inflammation (57).

The role of Ig in the pathogenesis of vasculitis has been controversial (6–8, 58). Vasculitis was not detected in mice lacking B cells, indicating that the vascular lesions were dependent on either the deposition of Ig or the presence of B cells. Definite, but relatively small, quantities of Ig were de-

tected within the vessel walls of diseased mice (when glomerular immune deposits were abundant; data not shown). This observation is consistent with Berden et al. (58), who found Ig within inflamed vessels but not within normal vessels of the same MRL-*lpr/lpr* mice. However, Moyer et al. (8) reported that perivascular lymphocytes and macrophages were present before vascular immune deposits; antibody deposition coincided with the development of necrotizing vasculitis in older MRL-*lpr/lpr* mice. These workers emphasized the cell-mediated nature of *lpr/lpr* vasculitis. However, they did find B cells in the outer zone of the inflammatory infiltrate. Cerny et al. (28) observed that anti-IgM treatment of C57BL/6-*lpr/lpr* mice ameliorated nephritis, but did not affect the vasculitis, suggesting, in contrast to our results, that B cells/Ig are not required for the development of vasculitis. It is difficult to reconcile the differences observed between JhD mice and anti-IgM treatment. The presence of low levels of B cells, heterologous rabbit antiserum, and presumably, immune complexes in the anti-IgM–treated animals may have been sufficient to allow for the development of vasculitis. These potential artifacts were not a problem in the JhD mice. Interestingly, previous work suggests that the role of B cells in vasculitis may differ from that in glomerulonephritis. Genetic (59) and passive autoantibody injection studies (18) have suggested that vasculitis and glomerulonephritis can occur and be induced separately, implying that they arise by distinct mechanisms. However, further work will be required to distinguish the underlying mechanisms.

Aside from its effect on nephritis, it is interesting that a lack of B cells was associated with reduced accumulation of T cells, which was prominent in the older cohort of mice. It has been proposed by Huang et al. (60) that activation of CD4$^+$ and/or CD8$^+$ SP cells is required for accumulation of DN T cells. Based on the present data, we propose that B cell interaction is an important pathway for such activation. Accumulation of both DN and SP T cells was reduced in mice lacking B cells, suggesting that B cells either interact equally with these subsets to promote their accumulation or else interact with a precursor that contributes equally to these T cell subsets. T cell accumulation is not, however, completely abrogated in the absence of B cells, especially in young animals. This suggests that other cell types, perhaps dendritic cells or macrophages, are also competent to activate precursors of accumulating T cells. The role of B cells in promoting further accumulation would become more apparent as macrophages and dendritic cells become more limiting amid the accumulating T cells in mice lacking B cells. The requirement for B cells to induce maximal lymphoaccumulation further suggests that the *fas* defect prevents an apoptotic event, which would normally occur only after T cell interaction (either stimulatory or tolerogenic) with an APC. Whether the *fas* deficiency is also required in the B cell in order to promote T cell accumulation could be tested via reconstitution of B cells in this model system.

In contrast to the periphery, DN T cells were disproportionately overrepresented in the thymi of *lpr/lpr* mice that had B cells. This curious observation could be explained by either of two mechanisms. First, DN T cells may be generated in the periphery and home to the thymus. The difference between B cell–sufficient *lpr/lpr* mice and those lacking B cells would then be attributed to a difference in the overall number of DN T cells, which in the mice lacking B cells would be two small to permit an "overflow" into the thymus. However, young *lpr/lpr* mice lacking B cells have as much lymphadenopathy as the B cell–sufficient mice, yet have a lower proportion of thymic DN T cells. Alternatively, B cells may directly affect the generation of some DN T cells in the thymus. This is plausible since small numbers of B cells are present in thymus (61). As in the periphery, a lack of B cells required for DN T cell expansion would limit DN T cell generation in the thymus of mice lacking B cells.

An assumption inherent in our conclusions is that the phenotypes of homozygous JhD-mutant mice are the result of the lack of B cells. An alternative interpretation is that a genetic locus linked to the JhD mutation on chromosome 12, rather than the mutation itself, is responsible for our findings. A candidate for such a locus, mapped by Watson et al. (62) to chromosome 12, modifies *lpr/lpr* renal disease in the MRL background. Although this locus is linked to the Jh locus (most likely ~40 cM away), it is unlikely that it—rather than the Jh knockout itself—is responsible for the present observations. The locus described by these workers is a "modifying locus," which, along with another unlinked locus, has an effect on the "renal index" that accounts for only ~50% of the variance in renal disease. By contrast, the difference in nephritis in our studies was dramatic: disease was either severe or absent. Thus it is unlikely that this locus accounts solely for our observations. The observation that *lpr/lpr* mice with either one (i.e., JhD/+) or two (i.e., +/+) copies of the MRL-derived chromosome 12 had equally severe disease also supports this conclusion.

Very recently, Jevnikar et al. (63) have shown that MRL-*lpr/lpr* mice that cannot express class II MHC molecules do not get nephritis. The authors considered that nephritis was abrogated either because of blocking of autoaggressive CD4$^+$ T cell generation within the thymus or lack of T cell activation within the kidney in the absence of class II expression on renal cells. Class II knockout *lpr/lpr* mice lack CD4$^+$ T cells, autoantibodies and MHC expression on B cells and tissues, complicating interpretation of why these mice do not get nephritis. Mice lacking B cells, on the other hand, have CD4$^+$ T cells and can express MHC class II in tissues, yet still do not get nephritis. In light of our data, we suggest that in class II–deficient mice, suboptimal B cell activation because of lack of class II expression may also play a role in preventing nephritis in these mice. Indeed, the MRL-*lpr/lpr* class II–deficient mice lacked serum autoantibodies (63). In addition, we would predict that, after restoration of CD4$^+$ T cells in these mice through cell transfer, disease would not ensue because of the inability to activate B cells. In any case, it is of great interest that in both class II–deficient *lpr/lpr* mice and JhD/JhD *lpr/lpr* mice, B cells are directly affected by the genetic alteration, and nephritis is prevented.

Our work underscores the importance of B cells in generating autoimmune-mediated tissue damage. The results indicate that B cells are critical for multiple components of

autoimmune-mediated inflammation, including those previously thought to be primarily mediated by humoral autoimmunity (glomerulonephritis), cellular autoimmunity (interstitial nephritis) or both (vasculitis). These data demonstrate that B cells could be an important target for therapy of systemic autoimmunity. Elimination of B cells or B cell subsets would have distinct advantages over removal of Ig alone (as in plasmapheresis). Pathogenic autoantibodies would be removed more efficiently than in plasmapheresis; moreover, B cell suppression would also ameliorate immune-mediated events requiring cell–cell interaction, such as interstitial nephritis and vasculitis, which would be unaffected by removal of Ig alone. A better understanding of the role of B cells and antibody in inducing autoimmune pathology will be necessary to design appropriate B cell–directed therapy.

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